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Citrus limonoids interfere with *Vibrio harveyi* cell–cell signalling and biofilm formation by modulating the response regulator LuxO

Amit Vikram,[1] Palmy R. Jesudhasan,[2] G. K. Jayaprakasha,[1] Suresh D. Pillai[1,2] and Bhimanagouda S. Patil[1]

Correspondence
Bhimanagouda S. Patil
b-patil@tamu.edu
Suresh D. Pillai
spillai@poultry.tamu.edu

[1]Vegetable and Fruit Improvement Center, Department of Horticultural Sciences, Texas A&M University, College Station, TX 77843, USA

[2]Food Safety and Environmental Microbiology Program, Texas A&M University, College Station, TX 77843, USA

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Citrus limonoids are unique secondary metabolites, characterized by a triterpenoid skeleton with a furan ring. Studies have demonstrated beneficial health properties of limonoids. In addition, certain citrus limonoids play a role in plant defence against insect pests. In the present study, five limonoids were purified from sour orange and evaluated for their ability to inhibit cell–cell signalling. The purified limonoids were tested for their ability to interfere with cell–cell signalling and biofilm formation in *Vibrio harveyi*. Isolimonic acid, deacetylnomilinic acid glucoside and ichangin demonstrated significant inhibition of autoinducer-mediated cell–cell signalling and biofilm formation. Furthermore, isolimonic acid and ichangin treatment resulted in induced expression of the response regulator gene *luxO*. In addition, *luxR* promoter activity was not affected by isolimonic acid or ichangin. Therefore, the ability of isolimonic acid and ichangin to interfere with cell–cell signalling and biofilm formation seems to stem from the modulation of *luxO* expression. The results suggest that isolimonic acid and ichangin are potent modulators of bacterial cell–cell signalling.

INTRODUCTION

Terpenoids are the largest group of plant secondary metabolites, and are suggested to act in a diverse array of plant physiological processes, including plant defence (Gershenzon & Dudareva, 2007; Langenheim, 1994). Several triterpenoids such as betulinic acid possess potent antimicrobial activity (Cowan, 1999). A unique class of triterpenoids, termed limonoids, is present in *Citrus* species. Early research focused on the antifeedant activity of citrus limonoids (Bentley *et al.*, 1990). However, in recent years, these secondary metabolites have been intensively investigated for their potential health benefits. Several reports from our laboratory (Poulose *et al.*, 2005; Tian *et al.*, 2001; Vanamala *et al.*, 2006) and by others (Guthri *et al.*, 2000; Miller *et al.*, 2004; Tanaka *et al.*, 2001) suggest a potential role for citrus limonoids in reducing the risks associated with various cancers. Limonoids are postulated to exert their effect by induction of glutathione *S*-transferase enzyme activity (Lam *et al.*, 1989; Perez *et al.*, 2009), apoptosis (Poulose *et al.*, 2005), antioxidant activity (Yu *et al.*, 2005) and suppression of cyclooxygenase 2/inducible nitric oxide synthase (Vanamala *et al.*, 2006).

Limonoids are distributed throughout the citrus plant, including the leaves, branches and fruit (McIntosh & Mansell, 1983), suggesting a possible defence function of these secondary metabolites in plants (McIntosh, 2000). Concurrent with this hypothesis, the antifeedant activity of citrus limonoids has been reported in several studies (Champagne *et al.*, 1992; Ruberto *et al.*, 2002). It has been suggested that increasing oxidation and skeletal rearrangement are associated with increased activity against insects. However, structurally simple limonoids present in the family *Meliaceae* are more potent insect repellents than complex citrus limonoids (Champagne *et al.*, 1992). Furthermore, limonoids isolated from the neem tree (*Azadirachta indica*, family *Meliaceae*) also demonstrate antibacterial activity (Atawodi & Atawodi, 2009). In contrast, reports elucidating the impact of citrus limonoids on micro-organisms are relatively limited. One study demonstrated the impact of limonin and nomilin on

Abbreviations: ACN, acetonitrile; AI-2, autoinducer-2; APCI, atmospheric pressure chemical ionization; CFS, cell-free culture supernatant; DCM, dichloromethane; EtOAc, ethyl acetate; DNAG, deacetyl nomilinic acid 17β-D-glucopyranoside; HAI, harveyi autoinducer; IOAG, isoobacunoic acid 17β-D-glucopyranoside; MeOH, methanol; qRT-PCR, quantitative RT-PCR.

Positive and negative ion mass spectra of citrus limonoids, and four-parameter sigmoidal models for the harveyi autoinducer, autoinducer-2 and *V. harveyi* BB120 biofilm formation, are available with the online version of this paper.

HIV-1 replication (Battinelli *et al.*, 2003). Furthermore, it has been suggested that citrus limonoids do not inhibit bacterial growth (Champagne *et al.*, 1992).

The elucidation of novel targets for drug development provides an opportunity to screen compounds that otherwise do not possess growth-inhibitory properties. A recently identified target is bacterial cell–cell signalling (also termed quorum sensing). Quorum sensing has been defined as coordinated gene expression in response to cell density (Bassler & Losick, 2006). Bioluminescence production in *Vibrio harveyi* is a quorum sensing-controlled process, and the various events in the signalling pathway have been well elucidated. Bioluminescence production in *V. harveyi* is controlled by three coincidence detectors that are part of a typical two-component pathway. The three coincidence detectors, LuxN, LuxPQ and CqsS, converge on the phosphorelay protein LuxU, which in turn activates the response regulator LuxO. LuxO activates the transcription of small antisense RNAs that regulate the production of luminescence by regulating the master regulator LuxR. Cell–cell signalling regulates various pathogenic processes in several bacteria (Erickson *et al.*, 2002; Sircili *et al.*, 2004; Walters & Sperandio, 2006). It is postulated that interference with bacterial cell–cell signalling attenuates bacterial pathogenicity (Rasmussen & Givskov, 2006). Consistent with this hypothesis, several synthetic and natural products have been demonstrated to interfere with cell–cell signalling and consequently to affect bacterial pathogenicity (Hentzer *et al.*, 2003; Manefield *et al.*, 2002; Persson *et al.*, 2005; Rasmussen *et al.*, 2005). Furthermore, plant secondary metabolites, such as halogenated furanones (Givskov *et al.*, 1996), polyphenols (Huber *et al.*, 2003) and organosulfur compounds from garlic (Persson *et al.*, 2005), have been reported to interfere with cell–cell signalling.

Grapefruit components, such as furocoumarins, limonoids and flavonoids, have been reported to interfere with cell–cell signalling (Girennavar *et al.*, 2008; Vikram *et al.*, 2010a, b). We were also interested in whether certain unique limonoids present in sour orange also possess the ability to interfere with bacterial cell–cell signalling. The present study provides evidence that certain citrus limonoids are potent inhibitors of cell–cell signalling.

METHODS

Materials. Reagent and HPLC-grade methanol (MeOH), ethyl acetate (EtOAc), acetonitrile (ACN), dichloromethane (DCM), molecular biology-grade DMSO and TLC silica gel 60F-254 plates were purchased from Fisher Scientific. All restriction enzymes and Deep Vent DNA polymerase were purchased from New England Biolabs.

Plant material. Sour orange (*Citrus aurantium* L.) fruits were collected from the Texas A&M University Kingsville Citrus Center, Weslaco, TX, USA. Seeds were separated from the fruits and air-dried under shade at 25 °C for 7–8 days to obtain ~2 % moisture level and were ground to powder (40–60 mesh size).

Extraction and purification of limonoids. Seed powder (2.0 kg) was extracted successively for 8 h each with hexane, EtOAc and MeOH in a Soxhlet apparatus. The EtOAc and MeOH extracts were filtered and concentrated under vacuum on a Rotavapor R-200 rotary evaporator (Buchi). The EtOAc extract (30 g) was dissolved in 500 ml DCM and extracted with 250 ml water. The vacuum-dried DCM fraction (28.9 g) was chromatographed on a silica gel column (300 g) with a stepwise linear gradient of EtOAc in DCM. Compounds 1A, 1B and 1C were eluted with DCM/EtOAc (92.5 : 7.5, 85 : 15 and 80 : 20, respectively). Yields of compounds 1A, 1B and 1C were 413, 805 and 24 mg, respectively.

The MeOH extract was processed according to our previously published method (Jayaprakasha *et al.*, 2006). The first column was packed with Dowex H^+ resin and the second was packed with Sepabeads adsorbant resin SP 70 (Sigma-Aldrich). The two columns were connected in tandem and vacuum-dried MeOH extract was loaded on the Dowex resin column. The column was washed thoroughly with deionized water. The wash from the Dowex column was passed over a Sepabeads resin column to adsorb limonoids. The Sepabeads column was later separated from the Dowex column and eluted with a stepwise linear gradient of ACN in water. Compounds 1D and 1E were eluted with 12.5 : 87.5 and 15 : 85 ACN/water, respectively. Fractions containing compound 1D in higher concentration were pooled and concentrated to yield 1.47 g of colourless crystalline product. The fractions with higher concentrations of compound 1E were pooled, dried under vacuum and redissolved in water/ACN (1 : 1) to yield 350 mg of colourless compound.

Analysis. All the fractions and purified compounds were analysed by TLC and HPLC. Fractions and purified limonoids were chromatographed on silica gel 60F-254 plates (Fisher Scientific) and detected with Ehrlich's reagent (2 % *N*,*N*-dimethylaminobenzaldehyde in ethanol), as described previously (Maier & Grant, 1970; Vikram *et al.*, 2010b).

HPLC was conducted according to a previously published method (Vikram *et al.*, 2007). Briefly, all the column fractions and purified limonoids were filtered through a 0.45 μm pore-size filter and chromatographed on a C_{18} Phenomenex Gemini series column (5 μm particle size, 250 × 4.6 mm). The limonoids were detected at 210 nm with a Diode Array detector 235C (Perkin Elmer). The gradient mobile phase was 3 mM phosphoric acid and ACN.

Identification of compounds and preparation of stock solution. Identities of the purified compounds were confirmed by atmospheric pressure chemical ionization (APCI)/electrospray ionization MS. The spectra of the purified compounds were compared with published data (Manners *et al.*, 2000). All the limonoids were dissolved in DMSO at a concentration of 20 mg ml^{-1}.

Bacterial strains and media. *V. harveyi* strains BB170 (*luxN*::Tn*5*), BB886 (*luxPQ*::Tn*5*), BB120 (wild-type), JAF483 (*luxO* D47A), JAF553 (*luxU* H58A), BNL258 (*hfq*::Tn*5lacZ*) and JAF548 (*luxO* D47E, black) were kindly provided by B. L. Bassler (Princeton University, Princeton, NJ, USA) (Freeman & Bassler, 1999a, b; Lenz *et al.*, 2004; Surette & Bassler, 1998). *Escherichia coli* #5, an environmental isolate (Qin *et al.*, 2004), was used as a positive control for autoinducer-2 (AI-2) activity. Autoinducer bioassay (AB) or Luria Marine (LM) media were used to culture the *V. harveyi* strains (Lu *et al.*, 2004; Surette & Bassler, 1998).

Plasmids. The molecular biology techniques were used as described by Sambrook & Russell (2001). The chloramphenicol-resistance (Cml^R) gene was amplified from vector pBAD33 and cloned into vector pFZY1 by partial digestion with *Pst*I, and the resultant vector was termed pAV09. Next, the *luxR* promoter (+400 to −56) was cloned into pAV09, resulting in plasmid pAV10. DNA primers for the *luxR* promoter were designed with a one-base modification to

generate *Eco*R1 and *Hin*dIII restriction sites. The fragment was amplified by PCR using Deep Vent DNA polymerase and cloned into plasmid pAV09 ahead of *lacZ* to generate plasmid pAV10. *V. harveyi* BB120 was transformed with pAV10 by electroporation at 2100 V (Sambrook & Russell, 2001). The transformed colonies were selected and maintained on agar plates supplemented with Cml (10 μg ml^{-1}).

Furthermore, *luxU*, *luxO* and *hfq* were amplified with primers carrying restriction sites for *Kpn*I, *Sal*I (*luxU*), *Sma*I, *Pst*I (*luxO*) and *Sac*I, *Sal*I (*hfq*). The amplified fragments were digested with the respective pairs of restriction enzymes and cloned into pBAD33. The new plasmids were termed pAV05 (*luxU*), pAV06 (*hfq*) and pAV07 (*luxO*). The plasmids were maintained in *E. coli* DH10B and electroporated into *V. harveyi* JAF553 (*luxU*), JAF483 (*luxO*) and BNL258 (*hfq*). The resulting strains were then termed AVS40 (JAF483 + pAV07), AVS41 (BNL258 + pAV06) and AVS42 (JAF553 + pAV05).

Growth and metabolic activity. Overnight cultures of *V. harveyi* BB120 were diluted 100-fold in AB media and exposed to limonoids (100 μg ml^{-1}) or an equivalent volume of DMSO. The cultures were grown for 16 h and OD_{600} was measured every 15 min by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments). The instrument was set to maintain a temperature of 30 °C and plates were constantly shaken at medium speed between readings. The data are presented as the mean of three biological replicates. To verify the results from the optical density experiment independently, the metabolic activity of *V. harveyi* in the presence of limonoids was measured by AlamarBlue (Invitrogen). The metabolic activity of *V. harveyi* was measured in a similar fashion as a growth curve with the addition of 25 μl AlamarBlue, and A_{570} and OD_{600} were monitored. The mean percentage reduction of AlamarBlue (Wang *et al.*, 2009) from three biological replicates was calculated and plotted against time.

Bioluminescence assay. The bioluminescence assay was carried out as described previously (Vikram *et al.*, 2010a). In brief, cell-free culture supernatants (CFSs) were prepared from *E. coli* #5 and *V. harveyi* BB120 by culturing in Luria–Bertani (LB) and LM media, respectively, to achieve high concentrations of autoinducer activity, followed by centrifugation at 10 000 r.p.m. for 10 min in a microcentrifuge. The supernatants were collected and filtered through a 0.2 μm pore-size membrane filter and stored at −20 °C. Inhibition of autoinducer [harveyi autoinducer (HAI) and AI-2]-mediated bioluminescence was measured in a 96-well plate assay (Lu *et al.*, 2004). The final concentrations of citrus limonoids tested were 6.25, 12.5, 25, 50 and 100 μg ml^{-1}. Diluted (5000-fold) overnight cultures (90 μl) of reporter strains BB886 (for HAI) and BB170 (for AI-2) were incubated with 5 μl CFS, 0.5 μl limonoids or DMSO, and 4.5 μl sterile AB medium at 30 °C with shaking at 100 r.p.m. Light production was measured by a Victor^2 1420 multilabel counter (Beckman Coulter) in luminescence mode. The values were recorded as relative light units and used in calculation (Lu *et al.*, 2004).

Overnight cultures of strains JAF553, JAF483, BNL258, AVS40, AVS41 and AVS42 were diluted 5000-fold and treated with isolimonic acid, ichangin or DMSO (control) (all at 100 μg ml^{-1}). One hundred microlitres of each was placed in a 96-well plate and grown for 4 h at 30 °C with shaking at 100 r.p.m. Luminescence was measured as described above. The relative light units from three biological replicates are presented as mean ± SD. In addition, OD_{600} values in the presence of DMSO, isolimonic acid and ichangin for strains JAF553 (0.38 ± 0.1, 0.36 ± 0.1 and 0.38 ± 0.07, respectively), JAF483 (0.33 ± 0.07, 0.32 ± 0.03 and 0.35 ± 0.02), BNL258 (0.27 ± 0.11, 0.26 ± 0.04 and 0.28 ± 0.03), AVS40 (0.24 ± 0.05, 0.25 ± 0.02 and 0.24 ± 0.18), AVS41 (0.2 ± 0.08, 0.21 ± 0.04 and 0.2 ± 0.02) and AVS42 (0.28 ± 0.07, 0.27 ± 0.13 and 0.28 ± 0.05) at the time of measurement of luminescence were recorded.

Biofilm assay. The biofilm assay was conducted as described previously (Vikram *et al.*, 2010a). An overnight culture of *V. harveyi* BB120 was diluted 1 : 50 in LM medium and 190 μl of this fresh culture was incubated with 9 μl sterile medium and 1 μl DMSO or limonoids (6.25, 12.5, 25, 50 or 100 μg ml^{-1}) dissolved in DMSO. *V. harveyi* JAF548 was used as negative control for the assay. The culture was placed in polystyrene 96-well plates (Fischer Scientific) and incubated at 26 °C for 24 h without shaking. Total biofilm mass was quantified by washing with phosphate buffer (0.1 M, pH 7.4), followed by staining with 0.3 % crystal violet (Fisher) for 20 min. The dye associated with biofilm was dissolved with 200 μl 33 % acetic acid, and A_{570} was measured. The mean ± SD of three biological replicates is presented.

RNA extraction and relative quantification of transcripts. An overnight culture of *V. harveyi* BB120 was diluted 100-fold with fresh AB medium, treated with isolimonic acid (100 μg ml^{-1}), ichangin (100 μg ml^{-1}) or an equivalent volume of DMSO, and grown further at 30 °C with shaking at 200 r.p.m. for 4 h. Bacterial cells were collected by centrifugation at 5000***g*** for 10 min. RNA was purified by using the RNeasy Mini Kit (Qiagen) and used as template for cDNA synthesis, as described previously (Vikram *et al.*, 2010a). For quantification of *qrr1*, RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer's instructions. For quantification, 25 ng cDNA was amplified with 10 pmol of primers and 10 μl SYBR Green (Applied Biosystems) for 40 amplification cycles (Vikram *et al.*, 2010a). All measurements were made on three biological replicates. Amplification of target sequences was done on an ABI Prism 7000 HT sequence detection system (Applied Biosystems). Relative quantification of target genes was calculated via the $2^{\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) and values were expressed as mean fold change (± SD). Primers (Table 1) were designed by Primer3 software (Rozen & Skaletsky, 2000).

***β*-Galactosidase assays.** An overnight culture of *V. harveyi* BB120 containing plasmid pAV10 was diluted 100-fold in AB medium. The diluted culture was treated with isolimonic acid, ichangin (both at 100 μg ml^{-1}) or an equivalent volume of DMSO, and grown for 4 h at 30 °C with shaking at 200 r.p.m. The β-galactosidase assay was performed as described by Miller (1972).

Statistical analysis. Inhibition of AI activity was calculated from the formula 100−[(relative AI activity/relative activity of positive control) × 100] (Lu *et al.*, 2004; Vikram *et al.*, 2010a) and is expressed as a percentage (mean ± SD). The percentage inhibition of biofilm formation was calculated as 100−[(A_{570} of sample well/A_{570} of positive control) × 100] and is expressed as mean ± SD. Non-linear regression analysis and *t* tests were conducted with SIGMAPLOT 11.0 (Systat Software).

The effects of different limonoids on each activity were analysed with an analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test on SPSS 16.0 (SPSS, Inc.). The effect was considered significant at $P<0.05$. The percentage inhibition and $\log_{10}$(concentration in μM) data from acyl homoserine lactone (AHL), AI-2 and biofilm assays were fitted to a four-parameter sigmoid model $y=y_0+a/\{1+\exp[-(x-x_0)/b]\}$ by using SIGMAPLOT 11.0. The IC_{90} and IC_{50} values were calculated from the model.

RESULTS

Purification and identification of citrus limonoids

Sour orange seed EtOAc extract yielded three aglycones. The compounds were analysed by HPLC according to Vikram *et al.* (2007) and found to be >96 % pure. The

Table 1. Primers used in the study

Gene	Forward or reverse primer	Sequence (5′–3′)
Real-time PCR		
hfq	Forward	ATGGCTAAGGGGCAATCTCT
	Reverse	ACTGTGGTGGCTCACTGGAC
luxO	Forward	GGCACTTTACCGCTCTTACC
	Reverse	GTCTGGGTGGCTTTTCTTCA
luxP	Forward	GATTTGACCACGCAGAAGG
	Reverse	CTTTCGCAGCATCATAGCC
luxQ	Forward	CGTTCACTCGGTTGTCTTCC
	Reverse	GGGCTTGGTCTTGCGATT
luxR	Forward	GTCAATGCCCTCAACCAAGT
	Reverse	TCTTCACGCCCAGTTTACG
luxS	Forward	AAAAGGAGACACCATCACGG
	Reverse	TGCTGCTCTGAAGGCGTT
qrr1	Forward	CCCCTCGGGTCACCTATCCAACT
	Reverse	GGCAGTCGGATCTATTGGCTCGTTC
rpoA	Forward	CACAGGGCCCGAGCCTGATTT
	Reverse	ACCACAGGGCCCGAGCCTG
Cloning		
luxR	Forward	CGAAGCCAAACTGCAAGAATTCGTTCTTCTGCA
	Reverse	TGTACCGATTGGAACAAGCTTGCGGAACAG
luxU	Forward	GCAAAATTCATGGTACCTGACATTATGACGG
	Reverse	TTACTGTCTAACTAGTCGACAAGCATTGAG
luxO	Forward	ACATCTAATGCCCGGGTGATTATGTCTACGA
	Reverse	ATCGCTACCAATTTCTGCAGACAGTTCTTCAA
hfq	Forward	AGACAGATGTGGGAGCTCTTAGATGGGGA
	Reverse	TGTGGGGATTGTCGACTGCCAGTCACAA
cml	Forward	ATGCCATAGCATTTTTATCC
	Reverse	GATTTAATCTGTATCAGG

identities of the purified compounds were confirmed by positive ion APCI-mass spectra for aglycones and negative ion APCI for glucosides. Compounds 1A, 1B and 1C were identified as ichangin (*m/z* 489, M+1), isoobacunoic acid (*m/z* 473, M+1) and isolimonic acid (*m/z* 471, M+1) (Fig. 1). The mass spectra of these compounds are presented in Supplementary Fig. S1. The MeOH extract yielded two glucosides, 1D and 1E, which were identified as deacetyl nomilinic acid 17β-D-glucopyranoside (DNAG) (*m/z* 669.3) and isoobacunoic acid 17β-D-glucopyranoside (IOAG) (*m/z* 651.2), respectively (Fig. 1). The negative ion mass spectra of DNAG and IOAG are presented in Supplementary Fig. S2. All the compounds were isolated as crystallized products.

Effect of citrus limonoids on cell growth and viability

Cell growth was measured by monitoring OD_{600} for 16 h. Limonoids did not affect growth of *V. harveyi* at 100 μg ml^{-1} (Fig. 2a). To validate the findings further, the cell viability of *V. harveyi* in the presence of 100 μg limonoids ml^{-1} was monitored for 16 h by using AlamarBlue. The results indicated no effect on cell metabolic activity by limonoids (Fig. 2b).

Inhibition of HAI- and AI-2-mediated bioluminescence by citrus limonoids

Bioluminescence produced by *V. harveyi* mutant strains was utilized as readout for HAI- and AI-2-mediated cell–cell signalling. All the tested compounds inhibited HAI- and AI-2-mediated bioluminescence in a concentration-dependent manner (Fig. 3). Three of the five tested limonoids, namely isolimonic acid, ichangin and DNAG, demonstrated >90 % inhibition of AI-2 activity at 100 μg ml^{-1}, and therefore IC_{90} values for AI-2 inhibitory activity were compared. In contrast, IC_{50} values were compared for HAI activity. Isolimonic acid was the most potent inhibitor of HAI and AI-2 activity (Fig. 3b and Supplementary Fig. S3) among the tested limonoids and inhibited 99.23 % of AI-2-mediated bioluminescence at 100 μg ml^{-1} (Fig. 3b). IC_{90} values for isolimonic acid, DNAG and ichangin for AI-2 activity were 38.90, 123.03 and 194.98 μM, respectively (Supplementary Fig. S3b). IC_{50} values for isolimonic acid, DNAG and ichangin against HAI activity were 148.59, 197.70 and 79.43 μM, respectively (Supplementary Fig. S3a). Isoobacunoic acid (Fig. 3c) had the lowest IC_{50} value of 65.01 μM against HAI activity; however, the four-parameter sigmoid plot demonstrated that the activity of isoobacunoic acid was saturated

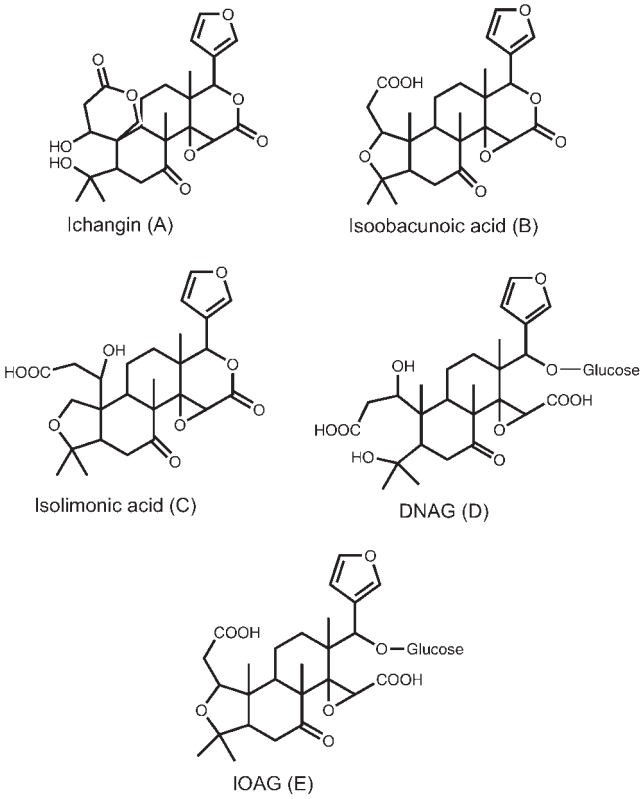

Fig. 1. Structures of the limonoids purified from sour orange in this study.

at $\geqslant$100 μM (Supplementary Fig. S3a). Ichangin (Fig. 3a) showed a more linear effect on HAI inhibition, and inhibited AI-2 activity by 9.63–91.58 % (Fig. 3a).

Inhibition of *V. harveyi* biofilm formation

Isolimonic acid was the most effective inhibitor of *V. harveyi* biofilm formation (Fig. 4 and Supplementary Fig. S4) with an IC_{50} value of 94.18 μM. IC_{50} values for ichangin, isoobacunoic acid, DNAG and IOAG could not be calculated, as these limonoids demonstrated $\leqslant$50 % biofilm inhibition under the experimental conditions. In comparison, the quorum-sensing-negative strain JAF548 showed very low levels of biofilm formation. Ichangin, isoobacunoic acid and DNAG demonstrated similar levels of inhibition on *V. harveyi* BB120 biofilm formation, whereas IOAG was the least effective in inhibiting *V. harveyi* biofilm formation (Fig. 4 and Supplementary Fig. S4).

Effect of limonoids on constitutively luminescent *V. harveyi* mutants

Because all the tested limonoids demonstrated inhibitory activity in HAI and AI-2 under the experimental conditions, it is possible that these limonoids are non-specific inhibitors of cell–cell signalling. To test this hypothesis, the effects of the limonoids were investigated in the constitutively bioluminescent mutants JAF553, JAF483 and BNL258 (Freeman & Bassler, 1999a, b). Based on their potency in bioluminescence assays and structural characteristics, isolimonic acid and ichangin were selected for further study. Strains JAF553 and JAF483 carry point mutations in the *luxU* and *luxO* genes, rendering them defective in phosphorelay (Freeman & Bassler, 1999a, b), while BNL258 contains a Tn*5* insertion in the *hfq* gene, leading to a non-functional Hfq protein (Lenz *et al.*, 2004). We reasoned that a diminished luminescence in these strains would indicate interference with signal transduction downstream of the non-functional gene. Interestingly, isolimonic acid treatment enhanced luminescence production in LuxU

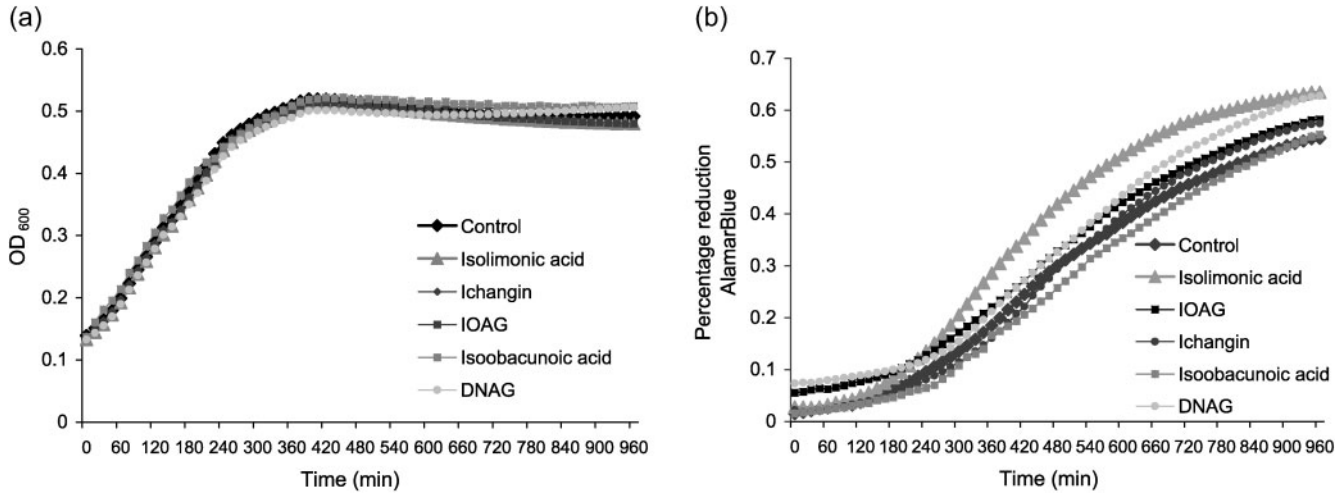

Fig. 2. (a) Growth curve of *V. harveyi* in the presence of limonoids (100 μg ml^{-1}). (b) Impact of limonoids on the metabolic activity of *V. harveyi* as measured by AlamarBlue reduction. DNAG, deacetyl nomilinic acid 17β-D-glucopyranoside; IOAG, isoobacunoic acid 17β-D-glucopyranoside.

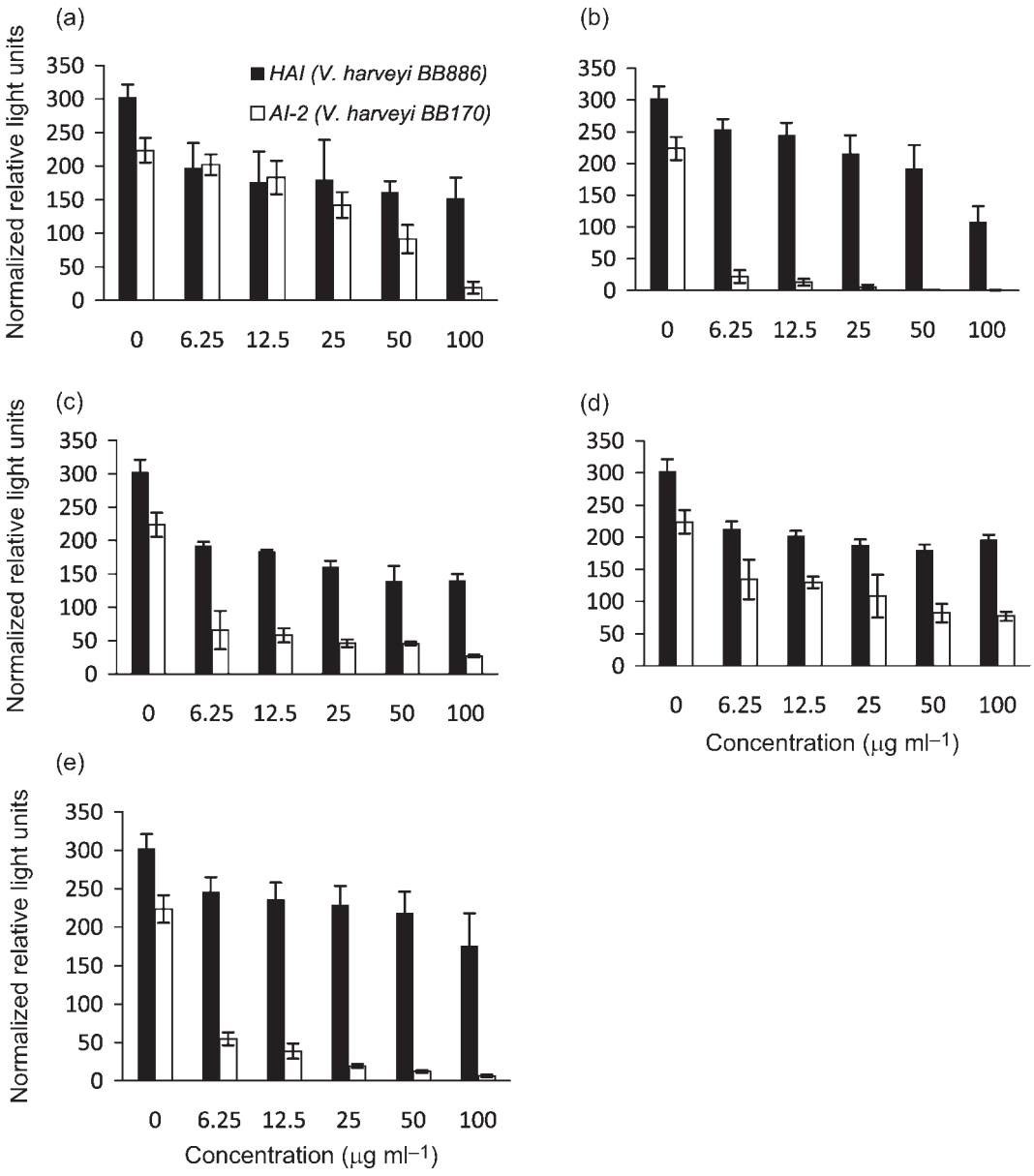

Fig. 3. HAI-1- and AI-2-induced bioluminescence in *V. harveyi* BB886 and BB170, respectively, in the presence of (a) ichangin, (b) isolimonic acid, (c) isoobacunoic acid, (d) IOAG and (e) DNAG. The relative light units for each strain were normalized by dividing by the value for the negative control (see text). The data are presented as normalized relative light units (mean ± SD).

(6.46 %) and LuxO (64.63 %) mutants significantly, but did not affect luminescence production in the *hfq* mutant (Fig. 5). Similarly, ichangin treatment induced bioluminescence in strain JAF483 (17.64 %), but reduced the luminescence in LuxU and *hfq* mutants (Fig. 5). Furthermore, to determine whether the increased luminescence can be negated by expression of a functional copy of the mutant or disrupted gene, *luxO*, *luxU* and *hfq* were expressed *in trans* under the control of an arabinose promoter. Expression from the plasmid resulted in decreased levels of luminescence in the transformed strains. Bioluminescence levels for isolimonic acid- or ichangin-treated strains were not significantly different from those of the control. The results suggest that isolimonic acid and ichangin are likely to affect the signal transduction pathway at or downstream of LuxO but upstream of Hfq (Fig. 5).

Expression analysis of the signal transduction pathway

To further understand the enhanced bioluminescence observed in *luxO* and *luxU* mutants by isolimonic acid

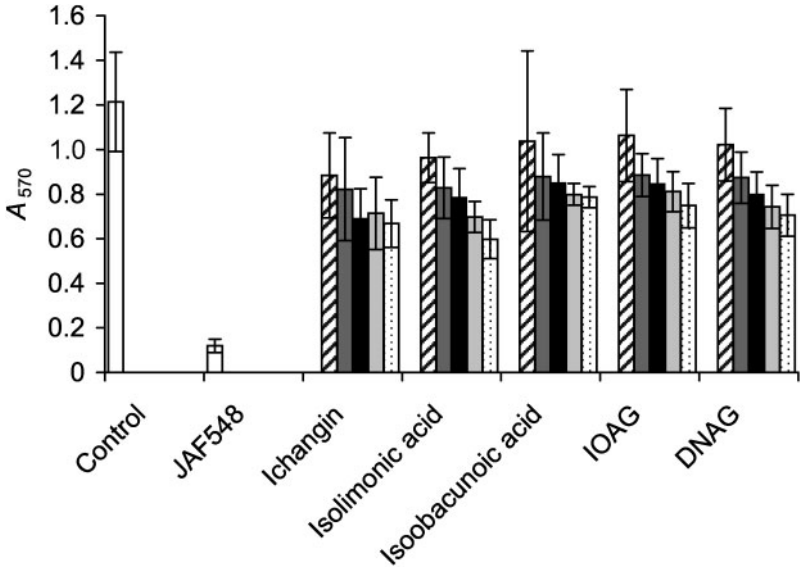

Fig. 4. Inhibitory activity of limonoids against *V. harveyi* BB120 biofilm. The *V. harveyi* biofilm was measured in the presence of limonoids at 6.25 (hatched bars), 12.5 (grey bars), 25 (black bars), 50 (light-grey bars) and 100 μg ml^{-1} (stippled bars). The data are presented as mean ± SD.

and ichangin, expression of *luxP*, *luxQ*, *luxO*, *hfq*, *luxR* and *luxS* was investigated in *V. harveyi* BB120 by using quantitative RT-PCR (qRT-PCR). Isolimonic acid treatment induced expression of *luxO* by 3.68-fold after 4 h (Fig. 6a). In addition, *hfq*, *luxP* and *luxQ* (2.53-, 2.42- and 5.29-fold, respectively) were also induced by isolimonic acid treatment. In contrast, *luxR* (−4.44-fold) was suppressed, while *luxS* (1.27-fold) was not affected by isolimonic acid. In the presence of ichangin, expression of *luxO*, *luxP*, *luxQ* and *hfq* was induced 23.7-, 3.54-, 2.64- and 2.53-fold (Fig. 6b), respectively. In addition, ichangin treatment resulted in a 2.4-fold suppression of *luxR*.

In wild-type *V. harveyi* strains, LuxO regulates levels of *luxR* mRNA by activating the transcription of sRNA (*qrr1–5*) (Lilley & Bassler, 2000; Tu & Bassler, 2007). Isolimonic acid and ichangin appeared to induce *luxO* but at the same time suppressed *luxR*. To confirm further that isolimonic acid and ichangin act by upregulating *luxO*, *luxR* promoter activity was measured. Isolimonic acid (Fig. 6c) and ichangin (Fig. 6d) treatment did not affect β-galactosidase activity. The empty vector was used as a control and did not demonstrate appreciable β-galactosidase activity. In addition, *qrr1* mRNA levels were quantified by using relative qRT-PCR. The *qrr1* gene was induced 8.7- and 12.3-fold by isolimonic acid and ichangin, respectively (Fig. 6e). These results further suggest that isolimonic acid and ichangin influence the cell–cell signalling pathway by modulating *luxO* expression.

DISCUSSION

In the present study, three limonoid aglycones and two limonoid glucosides were purified from sour orange seeds.

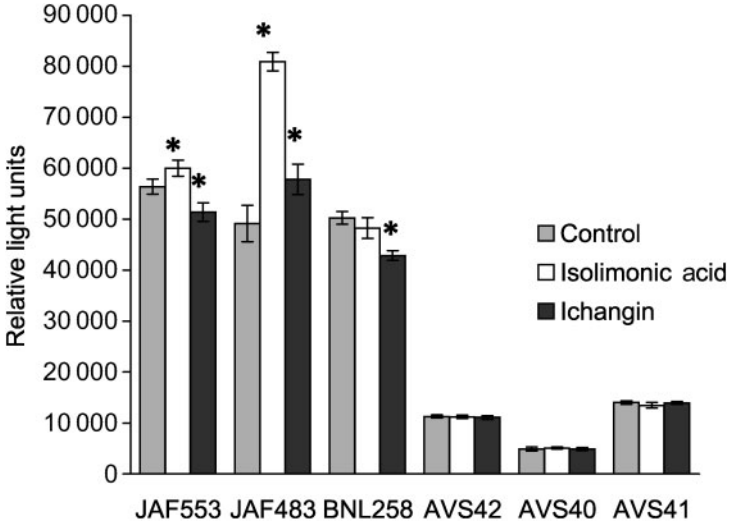

Fig. 5. Impact of isolimonic acid and ichangin (100 μg ml^{-1}) on bioluminescence of *V. harveyi* mutants JAF553 (*luxU* H58A), JAF483 (*luxO* D47A), BNL258 (*hfq*::Tn*5lacZ*), AVS40 (JAF483 complemented with *luxO*), AVS41 (BNL258 complemented with *hfq*) and AVS42 (JAF553 complemented with *luxU*).

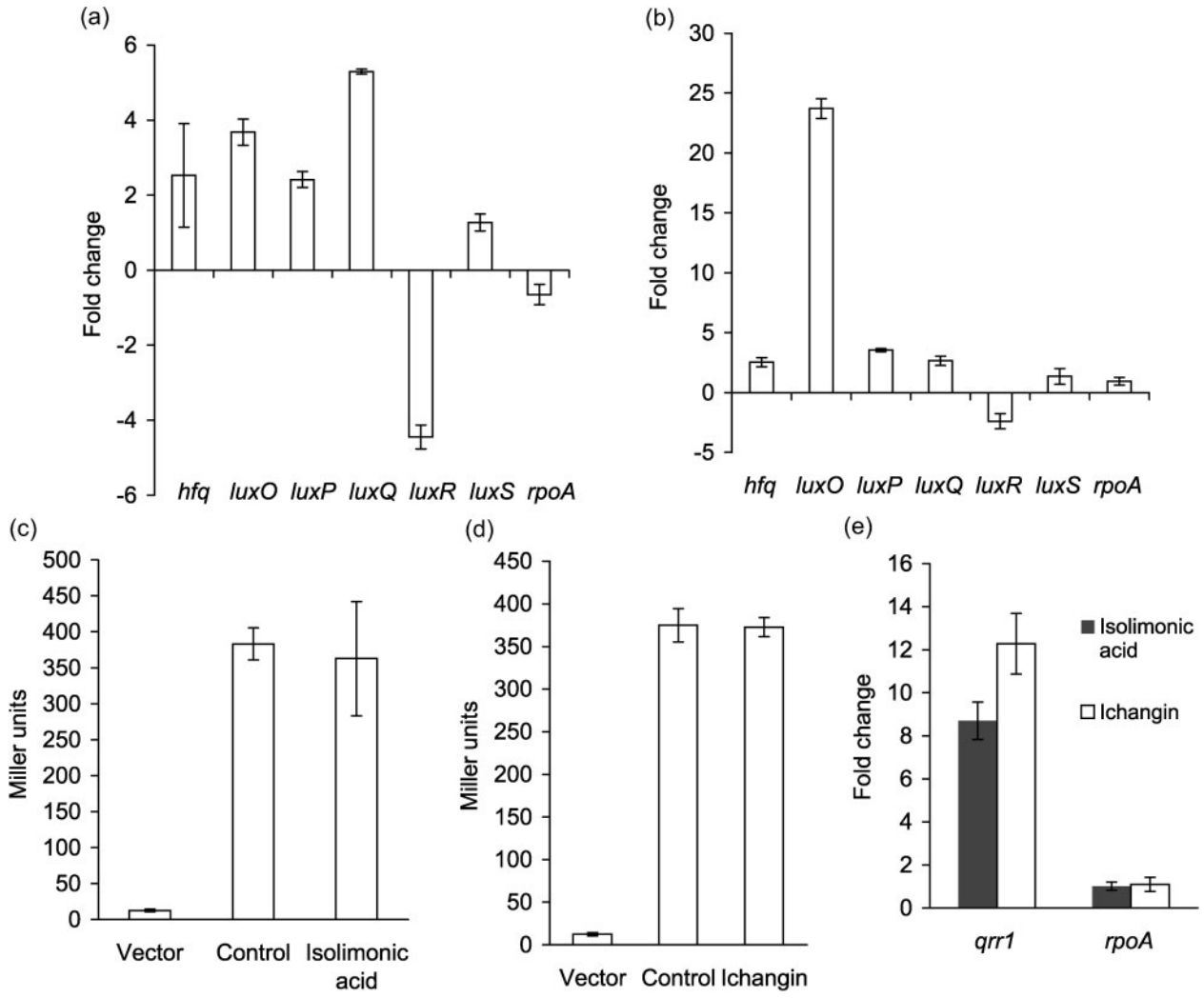

Fig. 6. Expression of the signal transduction pathway and AI-2 synthase in *V. harveyi* strain BB120 upon exposure (at 100 μg ml^{-1}) to (a) isolimonic acid and (b) ichangin, as measured by qRT-PCR. (c, d) LuxR promoter activity measured in *V. harveyi* BB120 in the presence of (c) isolimonic acid and (d) ichangin. (e) Relative quantification of *qrr1* in *V. harveyi* BB120, upon exposure to isolimonic acid and ichangin (at 100 μg ml^{-1}).

Despite their higher concentration in citrus juice, limonoid glucosides are difficult to purify owing to their very similar polarities. In addition, flavonoid glycosides, which are present at higher concentrations, interfere with the purification of limonoids. In the current study, we employed a cation H^+ exchange column. Flavonoid glycosides show weak ionization (Havsteen, 2002) and they are absorbed on the cation exchange column (Dowex), whereas limonoids are adsorbed on the Sepabeads column. The limonoid glucosides were eluted by using water and ACN to obtain DNAG and IOAG as crystallized products.

Bioluminescence production in *V. harveyi* is regulated by a two-component signal transduction pathway via three coincidence detectors (Henke & Bassler, 2004). The three coincidence detectors, LuxN, LuxPQ and CqsS, converge on the phosphorelay protein LuxU and share the rest of the pathway (Henke & Bassler, 2004). To measure HAI and AI-2 inhibitory activity, limonoids were tested in two *V. harveyi* strains carrying mutations in receptors. Strain BB886 is defective in cell signalling mediated by AI-2, whereas strain BB170 (LuxN::Tn*5*) does not detect *N*-hydroxybutanoyl-L-homoserine lactone (Bassler *et al.*, 1997). *E. coli* #5 does not produce acylhomoserine lactone (corresponding to HAI), but produces AI-2. Addition of CFS from *E. coli* #5 in the AI-2 activity bioassay ensured that the measured activity was due to the presence of AI-2. Furthermore, the reporter strains BB886 and BB170 produce and sense their own autoinducers. Therefore, the time to measure AI activity was chosen carefully. The assay was carried out until the negative control showed a luminescence reading of 100, which was taken as a final value and analysed.

Limonoids demonstrated a dose-dependent inhibition of HAI and AI-2 activity. To rule out that the observed effect was due to inhibition of bacterial growth, cell density (OD_{600}) in the presence of limonoids was measured. Citrus limonoids did not show growth-inhibitory effects on *V. harveyi* (Fig. 2), consistent with the study of Champagne *et al.* (1992). Furthermore, suppression of luminescence production suggests that limonoids act by a mechanism that mimics the phosphorylated LuxO or low-density phenotype. The LuxO mutant, locked in the phosphorylated condition, has been further shown to be defective in biofilm formation, indicating positive regulation of biofilm formation in *V. harveyi* by quorum sensing (Anetzberger *et al.*, 2009). Therefore, the biofilm formed by wild-type strain *V. harveyi* BB120 in the presence of various concentrations of limonoids was measured. Consistent with our hypothesis, a dose-dependent inhibition of biofilm formation was observed. In addition, isolimonic acid, which was the most potent inhibitor in the autoinducer assay, demonstrated significant inhibition of biofilm formation under the experimental conditions. This indicates that the tested limonoids inhibit biofilm formation, possibly by interfering with cell–cell signalling.

Isolimonic acid and ichangin were chosen for further investigation. Both demonstrated significant inhibition of HAI/AI-2 activity and biofilm formation. In addition, ichangin is structurally different from the other tested limonoids (Fig. 1a). We were interested to see whether isolimonic acid and ichangin differed in their mode of action. To determine the probable site of action, the effect of limonoids on constitutively luminescent *V. harveyi* mutants was investigated. Both isolimonic acid and ichangin enhanced luminescence production in the *luxO* mutant JAF483. The *luxO* gene in strain JAF483 contains a point mutation and is incapable of phosphorelay, and hence did not activate the transcription of sRNA (Freeman & Bassler, 1999b). Furthermore, isolimonic acid did not influence luminescence production in the *hfq* mutant BNL258. In contrast, ichangin treatment caused a small but significant reduction of luminescence in BNL258. The Hfq protein is non-functional in strain BNL258, and consequently *luxR* mRNA is stable and produces luminescence (Lenz *et al.*, 2004). Taken together, the data indicate that the possible site of action of isolimonic acid is between LuxO and Hfq, whereas ichangin seems to act at LuxO. To gain further insight, *luxO* mRNA transcript levels were measured by qRT-PCR (Fig. 6a, b). Induction of *luxO* suggested that isolimonic acid and ichangin modulate the signal transduction pathway by affecting *luxO* expression. Because LuxO regulates the stability of the *luxR* mRNA, higher expression of *luxO* will result in lower transcript levels of *luxR*. In addition, induction of *hfq* by isolimonic acid treatment was also observed. In wild-type *V. harveyi*, *luxO* in conjunction with the alternative sigma factor σ^{54} regulates the expression of the sRNAs *qrr1*, *qrr2*, *qrr3*, *qrr4* and *qrr5* (Lilley & Bassler, 2000; Tu & Bassler, 2007). In turn, these sRNAs, with the help of Hfq, destabilize the *luxR* mRNA (Lenz *et al.*, 2004). Consistent with these observations, an induction of *qrr1* and suppression of *luxR* mRNA transcript was observed in *V. harveyi* BB120 in the presence of isolimonic acid and ichangin. Together, higher expression levels of *luxO* and *hfq* plausibly explain the enhanced luminescence observed in *luxO* (JAF483) and *luxU* (JAF553) mutants. Furthermore, the induced expression of *luxPQ* may be a response to the induction of *luxO*. In addition, the results indicate that isolimonic acid did not

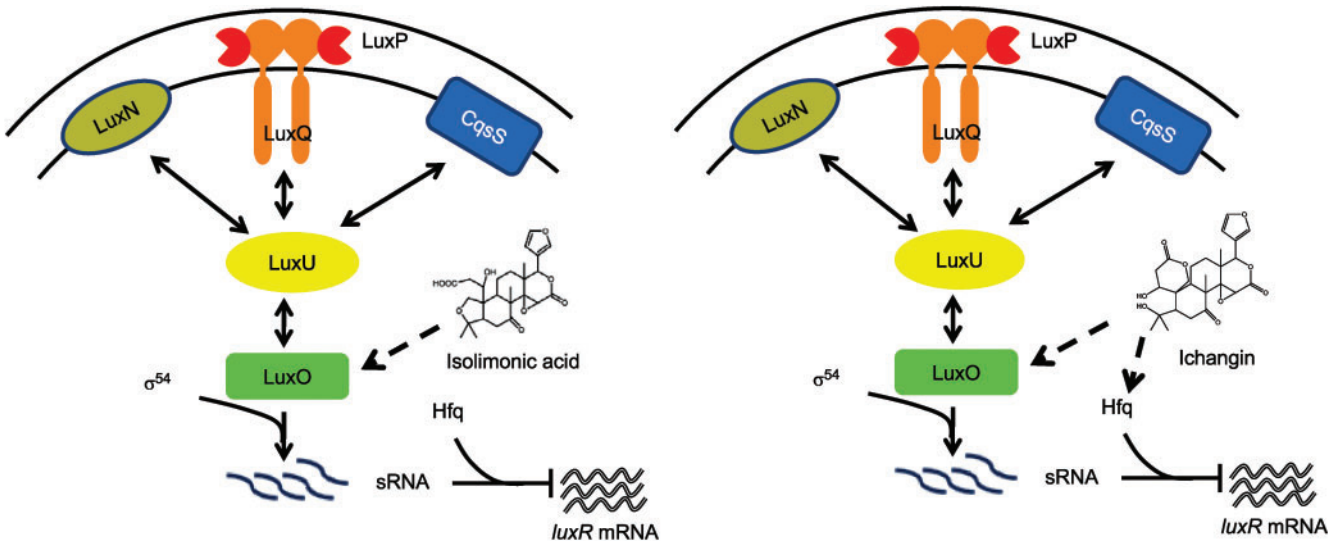

Fig. 7. Speculative model of the effect of isolimonic acid and ichangin on the signal transduction pathway in *V. harveyi*. The broken lines represent unknown modes of action on the expression of *luxO*. Figure adapted from Henke & Bassler (2004) and modified to depict the effect of the compounds.

affect AI-2 production, as *luxS* expression was unaltered under these experimental conditions. Interestingly, ichangin treatment also produced an expression profile similar to that of isolimonic acid, indicating a closely related mode of action. Like isolimonic acid, ichangin also seems to exert its action primarily by inducing *luxO* and *hfq* (Fig. 6b).

The quantitative PCR results suggest that isolimonic acid and ichangin treatment resulted in decreased *luxR* transcript levels. It is possible that the observed reduction in *luxR* transcripts is due to decreased transcriptional activity of *luxR* and not due to induced expression of *luxO*. To understand this further, we constructed a plasmid carrying the *luxR* promoter fused with *lacZ*, and measured *β*-galactosidase activity in the presence of isolimonic acid and ichangin. The results demonstrated that isolimonic acid and ichangin treatment did not affect *luxR* promoter activity. This observation suggests that the decreased level of *luxR* mRNA transcript level is due to induced expression of *luxO*. Together, the results suggest that isolimonic acid and ichangin interfere with *V. harveyi* cell–cell signalling by modulating *luxO* expression (Fig. 7). However, at this point it is not known whether this effect is direct or indirect. Furthermore, a negative feedback loop mechanism is suggested to regulate the levels of the transcriptional regulator HapR in *Vibrio cholerae* and to aid in switching from a high to a low cell density state (Svenningsen *et al.*, 2008). However, isolimonic acid and ichangin were only tested for their inhibitory effect under low-density conditions. Furthermore, the proposed mechanism (Fig. 7) seems to operate under low-density conditions. It is possible that isolimonic acid and ichangin have different effects at the transition phase from high to low cell density. During the early growth phase, LuxT has been suggested to regulate levels of *luxO* (Lin *et al.*, 2000), and it is possible that isolimonic acid and ichangin affect cell–cell signalling by affecting LuxT.

In conclusion, citrus limonoids, specifically isolimonic acid and ichangin, exhibited a strong inhibitory activity against bacterial cell–cell signalling as measured via the *V. harveyi* reporter strain model system. Furthermore, inhibition of quorum sensing-regulated processes, such as biofilm formation, was observed. Finally, isolimonic acid and ichangin appear to exert their effect by modulating the expression of the response regulator *luxO* and the RNA chaperone *hfq*. However, whether isolimonic acid and ichangin exert direct or indirect effects remains to be elucidated. The current study provides evidence that certain citrus limonoids possess the ability to interfere with bacterial cell–cell signalling in the model organism *V. harveyi*. However, the anti-quorum-sensing activity of citrus limonoids remains to be elucidated for human pathogens.

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